



User Manual

XF cell mito stress test kit

for research use only

XF96 Instructions



 *Seahorse Bioscience*
www.seahorsebio.com

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Preface

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Conventions

This guide uses the following conventions:

Convention	Type of Information
Bold	Indicates user interaction with elements of the software or system. Titles are spelled as they appear on the system.
1. 2. a. b.	Procedures are numbered and subprocedures are lettered. You must complete steps in the sequence they are presented to ensure success.
• •	Bullet lists indicate general information about a procedure. They do not imply a sequential procedure.

Information

HINT: Provides a helpful hint related to the current topic.

NOTE: Calls out a specific area of note in the protocol.

Related Documentation

In addition to the user manual, a Quick Start Guide is shipped with each kit.

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Overview of the Manual

- Section 1, “Overview of XF Cell Mito Stress Test Kit” on page 1 contains an overview describing the scientific merits of the stress test and a description of the kit contents and other required items.
- Section 2, “Reagent Preparation” on page 5 describes how to prepare and aliquot the reagents.
- Section 3, “Optimization Setup” on page 7 describes how to set up the optimization assay and software.
- Section 4, “Optimization Analysis” on page 13 describes how to analyze the results of the optimization assay.
- Section 5, “Stress Test Setup” on page 19 describes how to set up the XF cell mito stress test assay and software.
- Section 6, “Stress Test Analysis” on page 25 describes how to analyze the results of the XF cell mito stress test assay.

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1 Overview of XF Cell Mito Stress Test Kit

This section covers the following topics:

- Overview
- XF Cell Mito Stress Test Kit Contents
- Other Requirements

Overview

The ability to measure cellular metabolism and understand mitochondrial dysfunction has enabled scientists worldwide to advance their research in understanding the role of mitochondrial function in areas such as obesity, diabetes, aging, cancer, cardiovascular function, and safety toxicity.

Cellular metabolism is the process of substrate uptake, such as oxygen, glucose, fatty acids, and glutamine, and the subsequent energy conversion, through a series of enzymatically controlled oxidation and reduction reactions. These intracellular biochemical reactions result in the production of ATP and the release of heat and chemical byproducts, such as lactate and CO₂, into the extracellular environment.

Valuable insight into the physiological state of cells, and the alteration of the state of those cells, can be gained through measuring the rate of oxygen consumed by the cells, an indicator of mitochondrial respiration, the Oxygen Consumption Rate (OCR). Cells also generate ATP through glycolysis, the conversion of glucose to lactate, independent of oxygen. The measurement of lactic acid produced indirectly via protons released into the extracellular medium surrounding the cells, causing acidification of the medium, provides the Extracellular Acidification Rate (ECAR).

This assay is derived from a classic experiment to assess mitochondria, and serves as a framework with which to build more complex experiments aimed at understanding both the physiologic and pathophysiologic function of mitochondria, and to predict the ability of cells to respond to stress and/or insults.

The cells are metabolically perturbed by the addition of three different compounds in succession, that shift the bioenergetic profile of the cell (Figure 1.1). One group will serve as the control, with running media added as control “compounds”.

The first injection is Oligomycin (ATP Coupler). Oligomycin inhibits ATP synthesis by blocking the proton channel of the F₀ portion ATP synthase (Complex V). In mitochondrial research, it is used to prevent state 3 (phosphorylating) respiration. Within cells, it can be used to distinguish the percentage of oxygen consumption devoted to ATP synthesis and the percentage of oxygen consumption required to overcome the natural proton leak across the inner mitochondrial membrane.

The second injection is FCCP (ETC Accelerator). FCCP (Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone) is an ionophore that is a mobile ion carrier. FCCP is an uncoupling agent because it disrupts ATP synthesis by transporting hydrogen ions across the mitochondrial membrane instead of the proton channel of ATP synthase (Complex V). The collapse of the mitochondrial membrane potential leads to a rapid consumption of energy and oxygen, without the generation of ATP. In this case, both OCR and ECAR increase, OCR due to uncoupling, and ECAR due to the cells attempting to maintain their energy balance by using glycolysis to generate ATP.

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FCCP treatment can be used to calculate the spare respiratory capacity of cells, which is defined as the quantitative difference between maximal uncontrolled OCR, and the initial basal OCR. It has been proposed that the maintenance of some spare respiratory capacity, even under conditions of maximal physiological or pathophysiological stimulus, is a major factor defining the vitality and/or survival of the cells. The ability of cells to respond to stress under conditions of increased energy demand is influenced by the bioenergetic capacity of mitochondria. This bioenergetic capacity is determined by several factors, including the ability of the cell to deliver substrate to the mitochondria, and the functional capacity of the enzymes involved in electron transport.

The third injection is a combination of Rotenone (Mito Inhibitor B), a Complex I inhibitor, and Antimycin A (Mito Inhibitor A), a Complex III inhibitor. This combination shuts down mitochondrial respiration and enables both the mitochondrial and non-mitochondrial fractions contributing to respiration to be calculated. A decrease in OCR due to impaired mitochondrial function will occur, with a concomitant increase in ECAR, as the cell shifts to a more glycolytic state in order to maintain its energy balance.

Rotenone is a mitochondrial inhibitor that prevents the transfer of electrons from the Fe-S center in Complex I to ubiquinone (Coenzyme Q). This inhibition of Complex I prevents the potential energy in NADH from being converted to usable energy in the form of ATP.

Antimycin A, a complex III inhibitor, binds to the Qi site of cytochrome c reductase, thereby inhibiting the oxidation of ubiquinol in the electron transport chain of oxidative phosphorylation. The inhibition of this reaction disrupts the formation of the proton gradient across the inner membrane. Therefore the production of ATP is subsequently inhibited, as protons are unable to flow through the ATP synthase complex in the absence of a proton gradient.

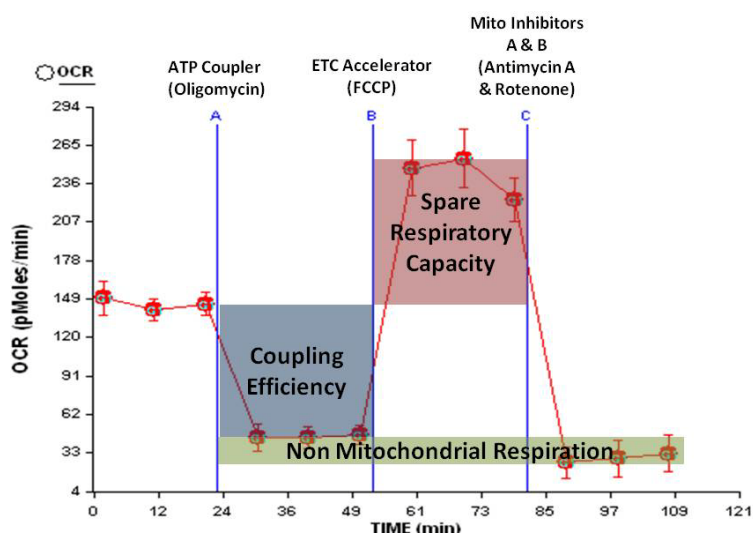


Figure 1.1 Example of the Stress Test

Basal respiration is predominantly controlled by the parallel re-entry pathways through the ATP synthase and leak. Addition of Oligomycin blocks the ATP synthase and the residual respiration is due to the proton leak. The decrease on adding Oligomycin approximates to the proton current flowing through the ATP synthase before the inhibitor was added. The decrease compared to basal provides the coupling efficiency. The

addition of a carefully calibrated concentration of the protonophore FCCP introduces a high artificial proton conductance into the membrane. This maximal respiration is now controlled by electron transport chain activity and/or substrate delivery. The increased respiratory capacity above basal respiration provides the spare respiratory capacity. Finally, electron transport chain inhibitors are added; any residual respiration is non-mitochondrial and needs to be subtracted from the other rates.

XF Cell Mito Stress Test Kit Contents

The XF Cell Mito Stress Test Kit contains enough reagents to run a mitochondrial profile experiment for 6 full XF96 plates. The kit contains the following reagents:

Reagent Name	Chemical Name
ATP Coupler	Oligomycin
ETC Accelerator	FCCP
Mito Inhibitor A	Antimycin A
Mito Inhibitor B	Rotenone
DMSO	Dimethyl Sulfoxide

Other Requirements

The following is a list of the required equipment and supplies not included in the XF Cell Mito Stress Test Kit:

- XF96 Extracellular Flux Analyzer
- Seahorse assay media (or equivalent) supplemented with fresh sodium pyruvate, glutamine, and glucose. (See the XF assay media product insert for more details.)
- XF96 FluxPak including cell plates, calibrant, and cartridges
- Serial dilution vessel (24-well plate or eppendorf tubes)
- Incubator - set to 37 °C, without CO₂
- Standard cell culture equipment:
 - Class II Biological Safety Cabinet
 - P10 - P1000 pipettes
 - Cell culture incubator
 - Bench top Vortex
 - Bench top mini-centrifuge

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2 Reagent Preparation

This section covers the following topics:

- Preparing the Reagents
- Aliquoting Reagents

Reagent Kit Contents



Figure 2.1 Kit Contents

One box of reagent compounds contains enough material for 6 full XF96 assays. The reagent box contains the following items:

- 4 vials of the reagent compounds in powder form (marked with a Seahorse logo):
 - Yellow top vial – ATP Coupler
 - Blue top vial – ETC Accelerator
 - Red top vial – Mito Inhibitor A
 - Green top vial – Mito Inhibitor B
- Grey top vial - 1 ml DMSO - used to resuspend the compounds
- 20 pre-labeled vials for aliquoting

Preparing the Reagents

This protocol describes the process of resuspending the compounds in the appropriate volume of DMSO to give a 2.5 mM stock concentration for each compound.

1. Remove the XF Cell Mito Stress Test Kit from the -20°C freezer and place on a lab bench for 30 minutes to thaw.

NOTE: When handling the compounds, nitrile gloves should be worn.

2. Once the reagent compounds are resuspended and aliquotated, they must be used within 6 weeks when stored at -20°C .
3. In a Class II Biological Safety Cabinet, resuspend powder reagent compound (marked with the Seahorse logo) in 180 μl of DMSO.
4. Vortex the vial, right side up and up side down, for 10 seconds each.
5. Spin down the vial in a mini centrifuge for approximately 5 seconds.
6. Repeat steps 1 through 4 to resuspend the other reagent compounds.
7. Write the date of resuspension in the Date Reconstituted box on the side of the XF Cell Mito Stress Test Kit.

Aliquoting Reagents

1. Open all the empty vials, of the same color, for one resuspended reagent (for example, all of the red topped vials).
2. Using a P-200 pipette, aspirate 30 μl of the resuspended reagent (in this example, the red vial containing the Mito Inhibitor A reagent).
3. Dispense 30 μl of reconstituted reagent into the appropriate empty vial; capping the vial immediately.
4. Repeat steps 1 through 3 for each resuspend reagent until each tube contains 30 μl of reagent.
5. Reagents that are not used immediately should be stored in the XF Cell Mito Stress Test Kit box at -20°C .

NOTE: Once the reagent compounds are resuspended and aliquotated, they must be used within 6 weeks when stored at -20°C .

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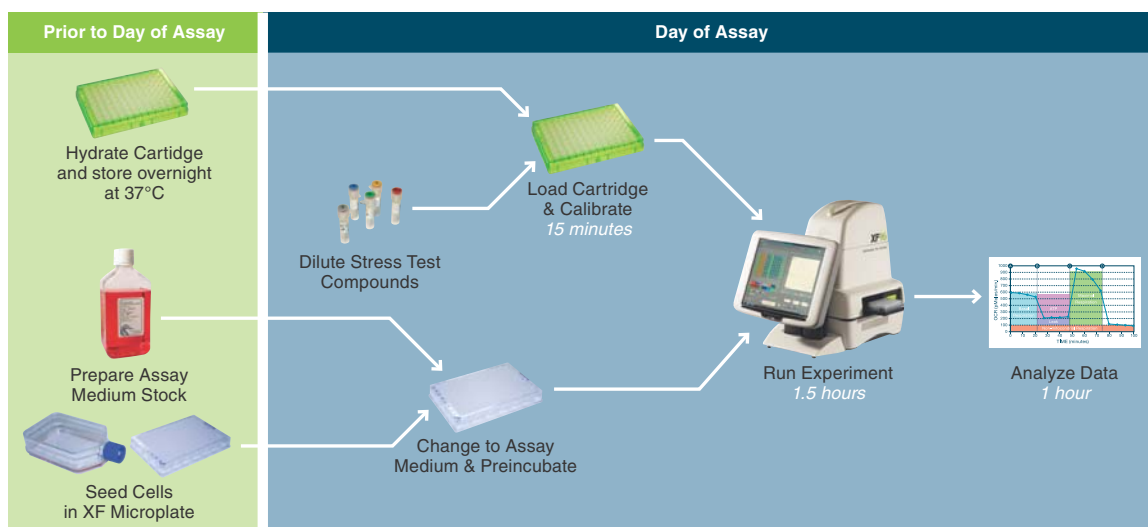
This section covers the following topics:

- Optimization Assay
- Cell and Cartridge Preparation
- Optimization Assay Example for XF96
- Setting Up the Optimization Software

Optimization Assay

The optimized working concentrations of the compounds used in the XF Cell Mito Stress Test Kit can change based on the cell line or cell type used for the assay. The Optimization Assay should be run for each compound when a new cell line is used.

Before running the optimization assay, the user should run a cell titration assay (described in the XF96 *Extracellular Flux Analyzer Users Manual*) to determine the proper cell seeding density for each cell line or cell type. Record the average basal OCR value for this cell density, as it will be used to define the protocol in the software wizard.



Assumptions

- The optimal cell seeding density has been determined prior to running the optimization assay.
- The average basal OCR was determined for the optimized cell seeding density.

Cell and Cartridge Preparation

The day before running the assay, do the following:

1. Hydrate the desired number of cartridges and store at 37 °C with no CO₂.
2. Prepare the desired number of cell plates by seeding cells at the appropriate density.

Optimization Assay Example for XF96

For this example, we will use a 24-well plate to do the dilutions. You can also use eppendorf tubes.

Preparing Compounds for Injection Ports

1. Remove the reagent box from the -20°C freezer.
2. Remove one tube for the reagent to be optimized and place the reagent box back in the freezer.

Creating a Serial Dilution

NOTE: To determine the volumes for the following procedure, use Table 3.1 Serial Dilution Volumes.

1. Perform serial dilution in a 24-well plate:
 - a. Prepare 100 µM top concentration by adding 20 µl from the appropriate reagent vial to 480 µl of Seahorse running media into well #1.
 - b. Dispense the appropriate volume of Seahorse running media into wells #2 through #10 (found in the **Seahorse Assay Medium** column in Table 3.1).
 - c. Using the pipette, mix up and down in well #1 several times.
 - d. Remove the appropriate volume of solution from well #1 (found in the **Dilution Volume** column in Table 3.1) and add to well #2.
 For example, for well #2 dilution, remove 300 µl from well #1 and add to well #2. For well #3 dilution, remove 500 µl from well #2 and add to well #3.
 - e. Using the pipette, mix up and down in well #2 several times.

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- f. Repeat steps c through d until all 10 wells have solution.

Table 3.1 Serial Dilution Volumes

Well/ Tube #	Injection Concentration (μ M) [10X final working concentration]	Final Working Concentration (μ M)	Seahorse Assay Medium (μ l)	Dilution Volume (from previous well/tube)
1	100	10	500*	0
2	30	3.0	700	300
3	15	1.5	500	500
4	12.5	1.25	167	833
5	10	1.0	200	800
6	7.5	0.75	250	750
7	5	0.5	333	667
8	3	0.3	400	600
9	1	0.1	667	333
10	0.3	0.03	700	300

NOTE: * For the ATP Coupler reagent, there could be solubility issues at the 100 μ M concentration. We recommend starting the dilution from the 30 μ M starting concentration to make serial dilutions (add 6 μ l of 2.5mM stock to 494 μ l Seahorse Running Media).

Refer to step 1a.

Preparing Cartridge and Cell Plate for Assay

1. See the plate map in Table 3.2 and dispense 25 μ l of injection medium with compound into port A of the appropriate cartridge.

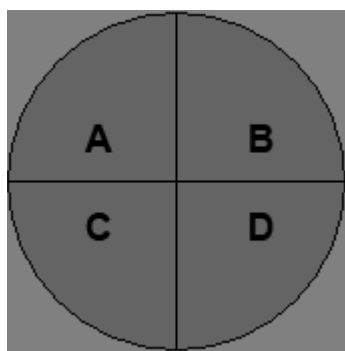


Figure 3.1 Well Plate Ports

Table 3.2 96-Well Final Working Concentration

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	0.03	0.1	0.3	0.5	0.75	1	1.25	1.5	3	3	Blank
B	Blank	0.03	0.1	0.3	0.5	0.75	1	1.25	1.5	3	3	Blank
C	Blank	0.03	0.1	0.3	0.5	0.75	1	1.25	1.5	3	3	Blank
D	Blank	0.03	0.1	0.3	0.5	0.75	1	1.25	1.5	3	3	Blank
E	Blank	0.03	0.1	0.3	0.5	0.75	1	1.25	1.5	3	10	Blank
F	Blank	0.03	0.1	0.3	0.5	0.75	1	1.25	1.5	3	10	Blank
G	Blank	0.03	0.1	0.3	0.5	0.75	1	1.25	1.5	3	10	Blank
H	Blank	0.03	0.1	0.3	0.5	0.75	1	1.25	1.5	3	10	Blank

2. Place the cartridge with injections in a 37 °C incubator with no CO₂ until the assay is started.
3. Perform a medium change on the cell plate by removing the running medium from each well and replacing with Seahorse Assay Medium (described in detail in the *XF96 Extracellular Flux Analyzer User Manual*). Final volume for each well is 225 μ l.
4. Place the cell plate in a 37 °C incubator with no CO₂ for one hour prior to the assay.

NOTE: Seahorse recommends medium changes using the XF Prep Station to insure accurate final volumes. When using the XF Prep Station, the final volume should be set to 225 μ l.

Setting Up the Optimization Software

1. Open the XF software.

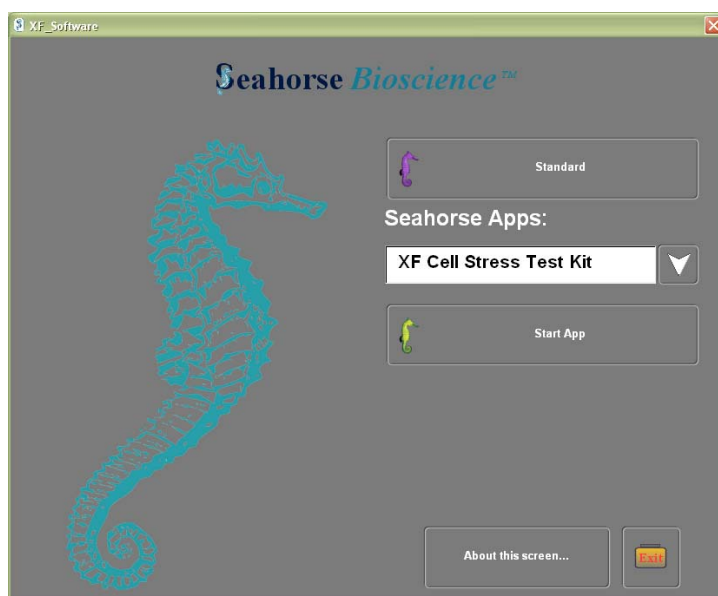


Figure 3.3 Seahorse Mito-Kit Screen

2. In the **Seahorse Apps** drop-down menu, choose **XF Cell Mito Stress Test Kit**.
3. Click the **Start App** button.
4. Click the **Run Optimization plate** button.

The **Optimization Injection Layout** screen appears.

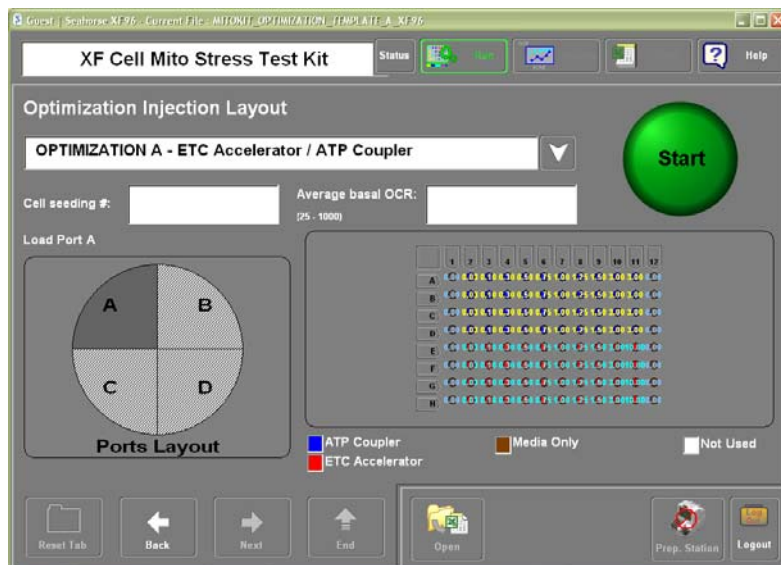


Figure 3.4 Optimization Assay – ETC Accelerator/ ATP Coupler Injection Map

5. On the injection map screen, do the following:
 - a. Choose the compound combination you are optimizing from the drop-down menu at the top of the page.
 - b. In the **Cell seeding #** box, enter the number of cells seeded per well.
 - c. In the **Average basal OCR** box, enter the average basal OCR for the seeded cell density.

NOTE: The average basal OCR value for the cell in question should have been determined prior to running the optimization assay when optimizing for cell seeding concentration.

6. Click the **Start**.
7. Choose a directory to save the file in, change the default file name, if necessary, and then click **OK**.
8. Place the cartridge and calibration plate with loaded injector ports on the sliding tray.
9. Click **Continue** to start calibration.
10. When prompted, replace the calibration (utility) plate with the cell plate.
11. Click **Start**.

12. The Optimization Assay will now run on the XF Analyzer.
13. When the run is over, follow the prompts in the software and remove the cartridge and cell plate and discard.

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4 Optimization Analysis

This section covers the following topics:

- Analyzing the Peak Response for the ETC Accelerator
- Determining the Peak
- Analyzing the Inhibitory Response for the ATP Coupler and Mito Inhibitor A and Mito Inhibitor B
- Determining the Inhibitory Concentration

Analyzing the Peak Response for the ETC Accelerator

When the ETC Accelerator is injected into the wells, the OCR response should increase in a dose dependent manner. This compound is the most sensitive in the kit to concentration changes, so it is important that the correct peak data point is chosen from the optimization experiment.

1. When the optimization experiment is complete, open the excel data output file, and choose the worksheet tab labeled 'Mito Stress Test Optimization' (see Figure 4.1).

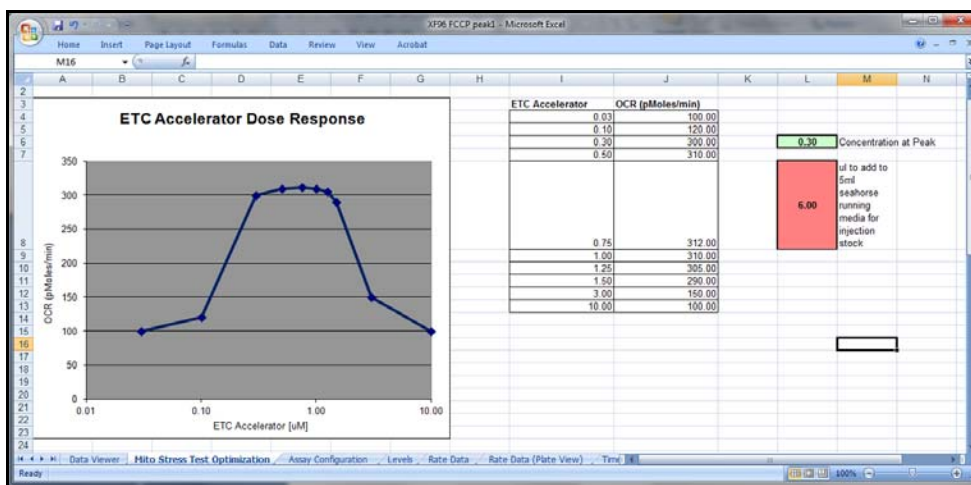


Figure 4.1
Screen shot of
the Mito Stress
Test
Optimization
excel output
for the ETC
Accelerator.

The graph on the left shows a dose response for the compound tested. The table in the middle shows the data used to generate the graph, compound concentration and OCR response. The software will calculate the peak response from the graph, in the green box labeled 'Concentration at Peak'. The user can change this concentration as needed to determine the proper volume of compound stock to add to 5ml Seahorse Assay Medium for the Cell Mito Stress Test.

2. The software automatically produces a dose response curve for the compound tested. This curve is shown with compound concentration on the x-axis and OCR on the y-axis.
3. The software will automatically suggest a concentration at the peak of the curve, but it is very important for the user to check that this is the true peak, and change accordingly.
4. When the peak concentration is determined, type it into the field labeled 'Concentration at Peak'.

- The software will automatically calculate the volume of 2.5 mM compound needed to make 5 ml of the final injection (the red box). Write this volume down in your notebook, and use this to prepare injections for the cell mito stress test.

Determining the Peak

In some situations, the peak response is not always obvious. The following presents a few examples of data from an optimization experiment, and how Seahorse recommends the peak concentration be chosen.

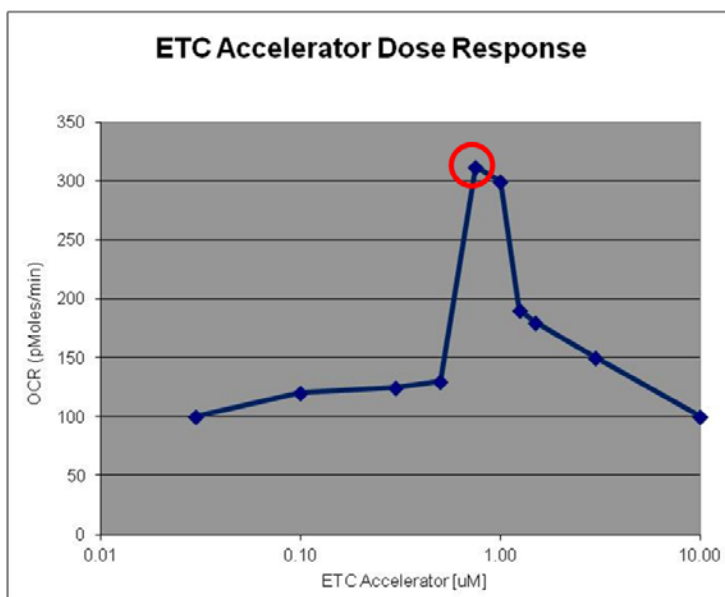


Figure 4.2 In this example the answer is obvious, the OCR values rise as ETC Accelerator concentration increases, and falls after the peak is reached. Here, the user should choose 0.75 uM as the optimized concentration.

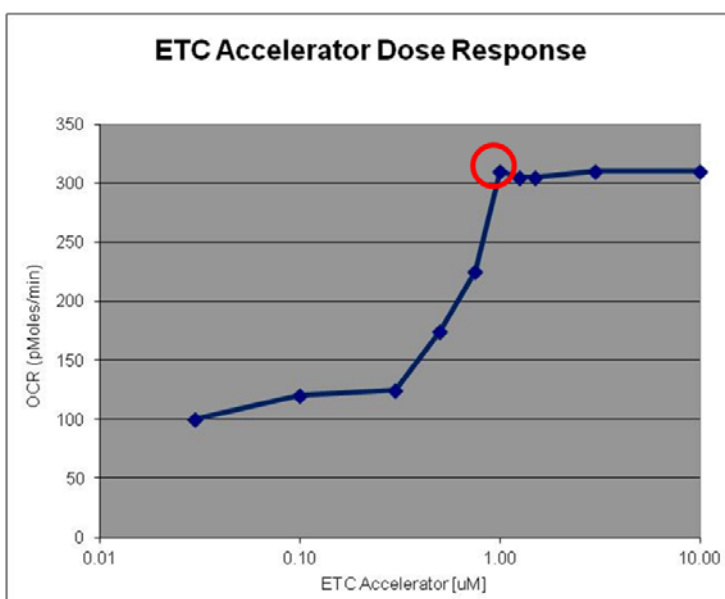


Figure 4.3 In some cases, the OCR response rises gently with concentration, and levels out once the peak is reached. In this example, the user should choose the concentration where the curve flattens out, in this case 1 uM.

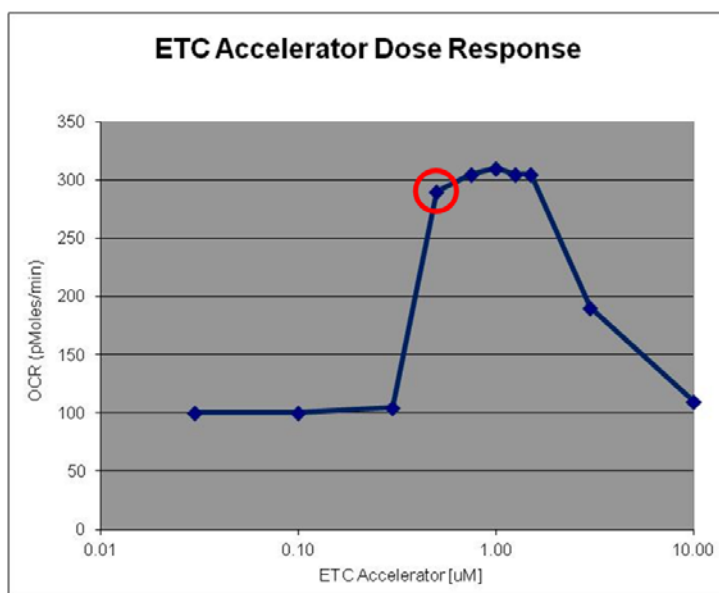


Figure 4.4 In this example, the curve shows a steep increase in OCR over a small range of concentrations. Even though there is a higher peak point at 0.75 uM, the user should choose the point after the steep increase, in this case 0.5 uM. The idea is to maximize the response in the lowest concentration possible.

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Analyzing the Inhibitory Response for the ATP Coupler and Mito Inhibitor A and Mito Inhibitor B

When the ATP Coupler or Mito Inhibitor A or Mito Inhibitor B are injected into the wells, the OCR response should decline in a dose dependent manner. These compounds should be run separately to determine the proper concentration for inhibition for each compound. As the responses are similar for these three compounds, the technique for analyzing the data is the same.

1. When the optimization experiment is complete, open the excel data output file, and choose the worksheet tab labeled 'Mito Stress Test Optimization' (see Figure 4.5).

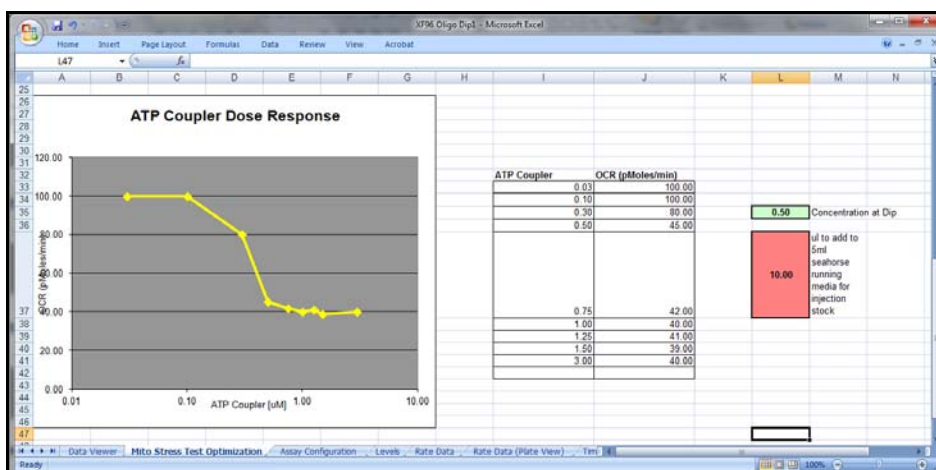


Figure 4.5
Screen shot of the
Mito Stress Test
Optimization excel
output for the ATP
Coupler.

The graph on the left shows a dose response for the compound tested. The table in the middle shows the data used to generate the graph, compound concentration and OCR response. The software will calculate the inhibitory response from the graph, in the green box labeled 'Concentration at Dip'. The user can change this concentration as needed to determine the proper volume of compound stock to add to 5 ml Seahorse Assay Medium for the Cell Mito Stress Test.

2. The software automatically produces a dose response curve for the compound tested. This curve is shown with compound concentration on the x-axis and OCR on the y-axis.
3. The software will automatically suggest a concentration at the inhibitory concentration of the curve, but it is very important for the user to check that this is the true maximal inhibition, and change accordingly.
4. When the inhibitory concentration is determined, type it into the field labeled 'Concentration at Dip'.
5. The software will automatically calculate the volume of 2.5 mM stock compound needed to make 5 ml of the final injection (the red box). Write this volume down in your notebook, and use this to prepare injections for the mito stress test.

Determining the Inhibitory Concentration

In some situations, the inhibitory response concentration is not always obvious. The following presents a few examples of data from an optimization experiment, and how Seahorse recommends the inhibitory concentration be chosen.

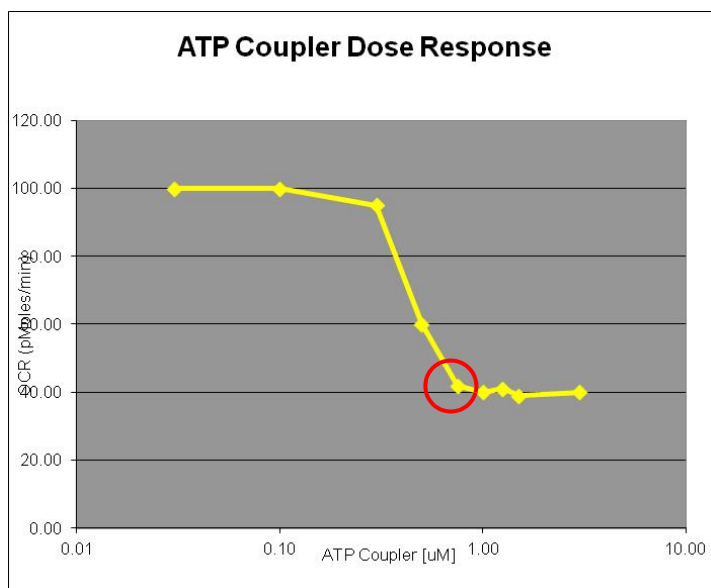


Figure 4.6 In this example the answer is obvious, the OCR values fall as ATP Coupler concentration increases, and flattens out once the nadir is reached. Here, the user should choose 0.75 uM as the optimized concentration.

NOTE: In this example, the curve trends subtly downward once the curve bottoms out. Here it is important that the user manually enter the 0.75 uM concentration in the green box in the spreadsheet, as the software may automatically recommend a higher concentration.

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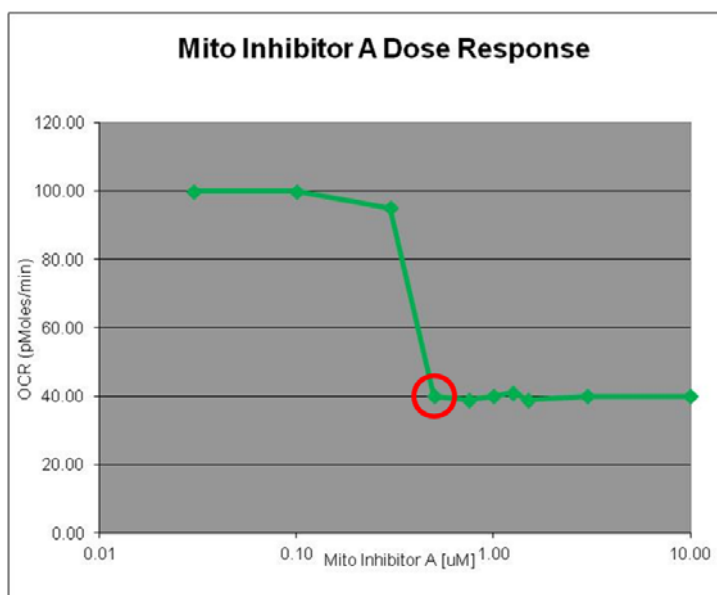


Figure 4.7 In this example, for Mito Inhibitor A, the curve shows a steep decrease in OCR over a small range of concentrations. Here, the proper concentration for the best inhibitory response is 0.5 uM.

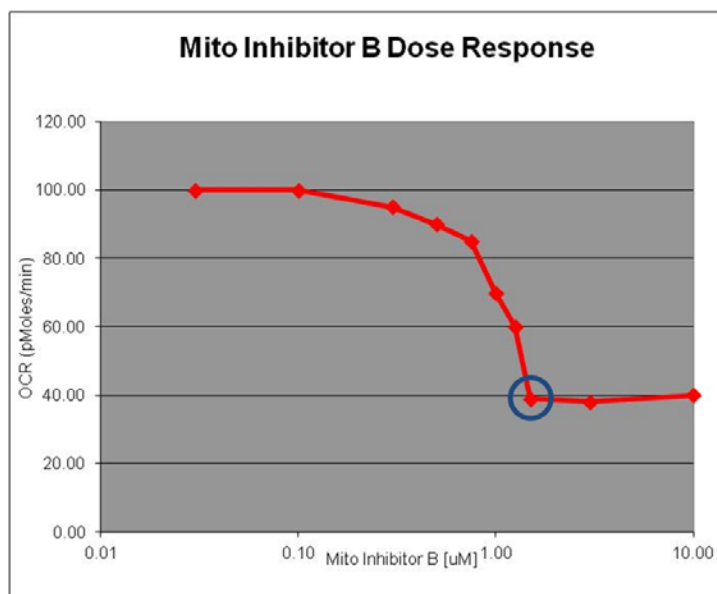


Figure 4.8 In some cases, like this one for Mito Inhibitor B, the curve shows a gradual decline in OCR over increasing concentrations. Again, the concentration where the curve flattens out at its nadir is the optimized inhibitory concentration. In this example that concentration is 1.5 uM.

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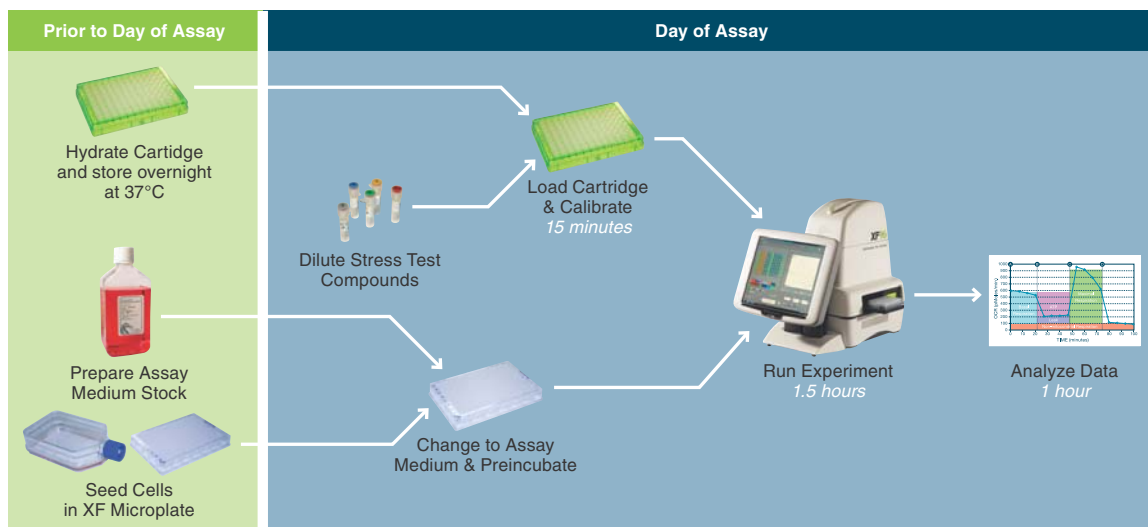
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5 Stress Test Setup

This section covers the following topics:

- XF Cell Mito Stress Test Assay
- Cell and Cartridge Preparation
- Preparing Reagents
- Setting Up the XF Cell Mito Stress Test Software

XF Cell Mito Stress Test Assay



Assumptions

- The optimal cell seeding density has been determined prior to running the optimization assay.
- The average basal OCR was determined for the optimized cell seeding density.
- The user has run the optimization assay for all 4 compounds for the cell line or cell type in question.

Cell and Cartridge Preparation

The day before running the assay, do the following:

1. Hydrate the desired number of cartridges and store at 37 °C with no CO₂.
2. Prepare the desired number of cell plates by seeding cells at the appropriate density.

Preparing Reagents

1. Determine the appropriate concentration of each injected compound by running the compound optimization experiments and analyzing the data.
2. Prepare 5 ml of each compound to be injected.
 - The Mito Inhibitor A and Mito Inhibitor B are added at the same time during the assay, so the user should prepare 1 5 ml injection stock with both compounds added.
 - The concentration of each stock compound is 2.5 mM.
 - The volume of the 2.5 mM stock to be used for preparing the 10x injection compound is automatically calculated in the “Optimization tab”.
 - If calculating manually, prepare a 10X injection concentration compared to the final desired working concentration.

NOTE: Seahorse recommends preparing 5 ml of each injection so the user can use a multichannel pipette reservoir. The actual volume needed to fill the 80 ports for injection is 2.0 ml. If the optimized concentration is too high to make the full 5 ml, recalculate to make 0.5 ml of injection stock.

Preparing Cartridge for Injection and Cell Plate

1. Add the appropriate volume of the prepared compound reagents into the appropriate injection port:
 - Port A - 20 µl - ATP Coupler
 - Port B - 22 µl - ETC Accelerator
 - Port C - 25 µl - Mito Inhibitor A and Mito Inhibitor B
2. Store cartridge in a 37 °C incubator with no CO₂ until ready to use.
3. Perform a medium change on the cell plate by removing the running medium from each well and replacing with Seahorse Assay Medium (described in detail in the *XF96 Extracellular Flux Analyzer User Manual*). Final volume for each well is 180 µl.
4. Place the cell plate in a 37 °C incubator with no CO₂ for one hour prior to the assay.

NOTE: Seahorse recommends medium changes using the XF Prep Station to insure accurate final volumes. When using the XF Prep Station, the final volume should be set to 180 µl.

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Setting Up the XF Cell Mito Stress Test Software

1. Open the XF software.

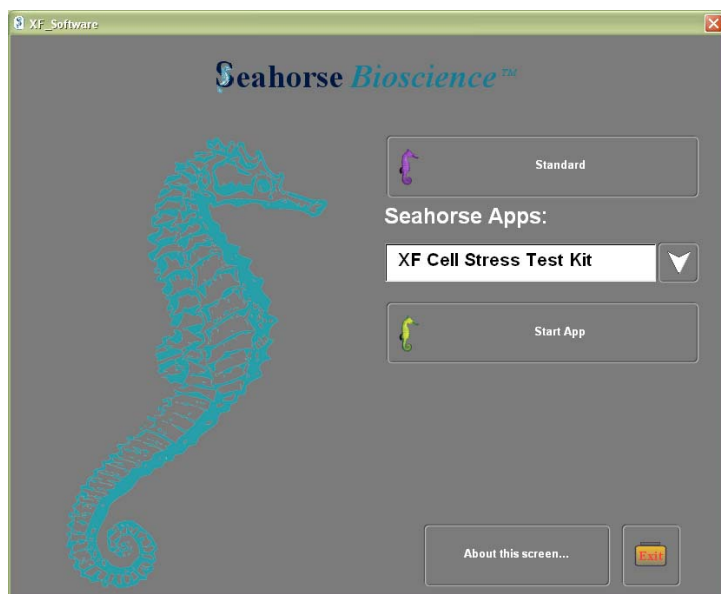


Figure 5.1 Seahorse Mito-Kit Screen

2. In the **Seahorse Apps** drop-down menu, choose **XF Cell Mito Stress Test Kit**.
3. Click the **Start App** button.
4. Click the **Run Stress Test** button.

The **XF Cell Stress Test Setup** screen appears.

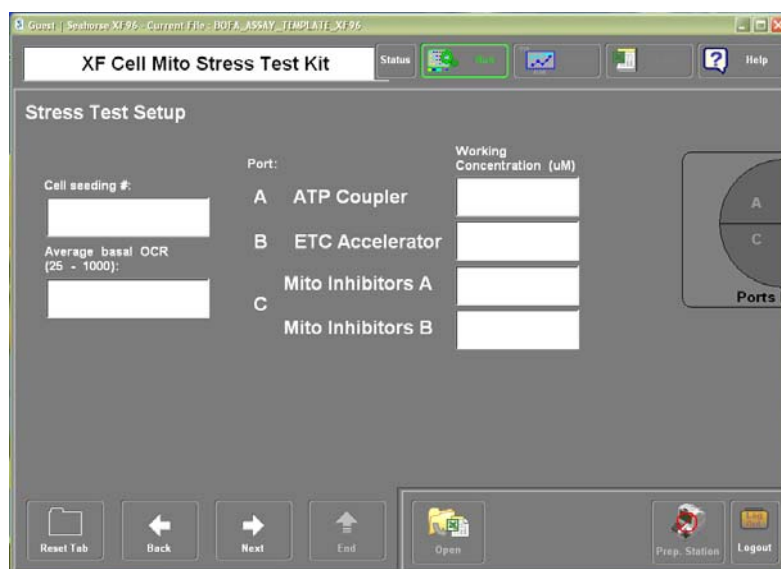


Figure 5.2 XF Cell Stress Test Setup Screen

5. Do the following:

- In the **Cell seeding #** box, enter the number of cells seeded per well.
- In the **Average basal OCR** box, enter the average OCR.

NOTE: The average basal OCR value for the cell in question should have been determined prior to running the optimization assay when optimizing for cell seeding concentration.

- Enter the final working concentration for each reagent that will be injected.

6. Click the **Next** button.

The group info screen appears.

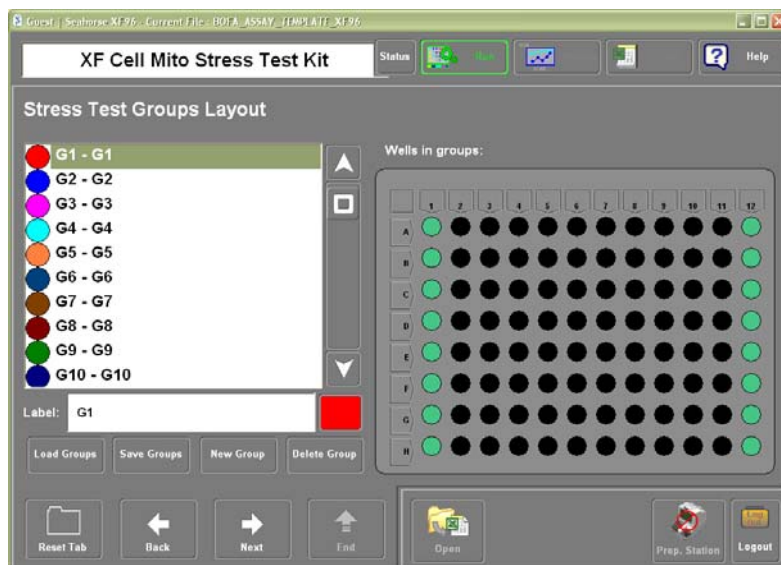


Figure 5.3 Group Info Screen

7. Assign a group to the unassigned wells by choosing a color and a name, and then clicking on the appropriate wells.

NOTE: The groups are defined as different treatments before the XF Cell Mito Stress Test is run. All wells will get the same compound injections when the stress test is run.

8. Click the **Next** button.

The **Stress Test Injection Layout** screen appears. This screen shows the injection layout for the Stress Test cartridge.

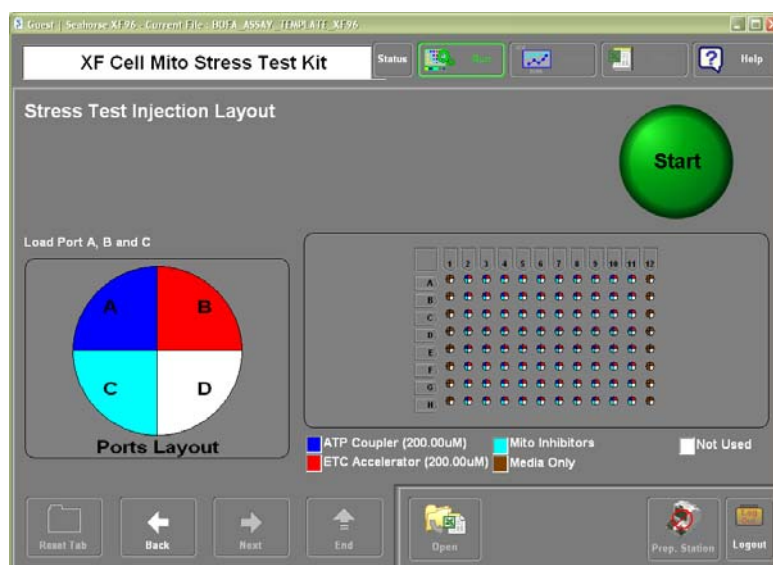


Figure 5.4 Stress Test Injection Layout Screen

9. Click **Start**.
10. Choose a directory to save the file in, change the default file name, if necessary, and then click **OK**.
11. Place the cartridge and calibration plate with loaded injector ports on the slide out tray.
12. When prompted, replace the calibration (utility) plate with the cell plate.
13. Click **Start**.
14. The Stress Test will now run on the XF Analyzer.
15. When the run is over, follow the prompts in the software and remove the cartridge and cell plate and discard.

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6 Stress Test Analysis

This section covers the following topics:

- Data Presentation
- Spare Respiratory Capacity
- Coupling Efficiency
- Basal Respiration
- ETC Accelerator Response
- ATP Coupler Response
- The Mitochondrial Proton Circuit
- Mitochondrial Proton Current (Respiration)

Since mitochondrial function can be analyzed in a number of different ways, one of the key advantages to using the XF Cell Mito Stress Test Kit is that key metrics are calculated automatically by the software, allowing for easy standardization between runs.

Upon completion of the XF Cell Mito Stress Test, the data outputs to a standard XF excel file. The standard analysis tabs are still present (Data Viewer, Rate Data, etc) as well as the troubleshooting tabs that contain the raw data (Levels, Calibration). When the XF Cell Mito Stress Test is run, an additional tab is present called the Mito Stress Test Output. Click on this tab to view the data calculated from this experiment.

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Data Presentation

Data from five separate metrics are automatically calculated by the XF Cell Mito Stress Test software and presented in the format shown in Figure 6.1.

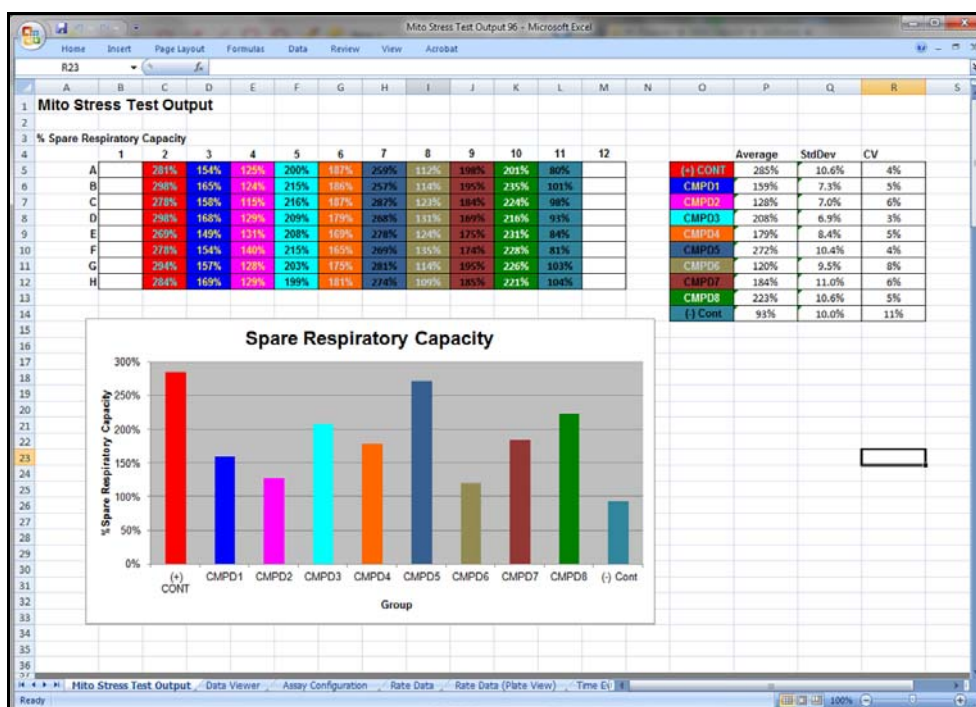


Figure 6.1
Example data
output for the
XF Cell Mito
Stress Test.

Data Table — The data table in the upper left-hand portion of the screen shows the calculated metric for each individual well. A user can scan the data quickly, and note if there are any outliers in individual groups. The groups previously chosen in the Stress Test Groups Layout screen, within the Stress Test wizard, are delineated by color in this table. Note that the color code is consistent throughout the results data, including the histogram.

Statistics Table — The statistics table in the upper right hand of the screen shows average, standard deviation, and coefficient of variation (CV) for each individual group. This provides the user an indication of the reproducibility between the groups.

Histogram — The graph on the bottom of the screen shows a histogram, with the metric of interest on the y-axis and the group on the x-axis. This graph provides the user a snapshot view of the group data as they relate to each other.

The data for the five output metrics are calculated according to Table 6.1.

Table 6.1 Description or formula for each output metric explaining calculation

Metric	Description/Formula
Spare Respiratory Capacity*	=ETC Accelerator Response/Basal Respiration
Coupling Efficiency*	=1 - (ATP Coupler Response/Basal Respiration)
Basal Response	The 3rd basal measurement (measurement before injection)
ETC Accelerator Response	The maximum rate after ETC Accelerator injection
ATP Coupler Response	The minimum rate after ATP Coupler injection

NOTE: * For the calculated metrics Spare Respiratory Capacity and Coupling Efficiency, the minimum Mito Inhibitor Response is subtracted from the injection responses to account for non-mitochondrial respiration.

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Spare Respiratory Capacity

Spare respiratory capacity is calculated by noting the OCR response to the ETC Accelerator, and dividing that number by the basal respiration and multiplying by 100 to get a percentage. The non-mitochondrial respiration is subtracted from both of these values. This is the true 'stress test' measure of the assay, as it provides the user an idea of a cells maximum ATP production, therefore cells with a higher capacity have a greater ability to respond to stress.

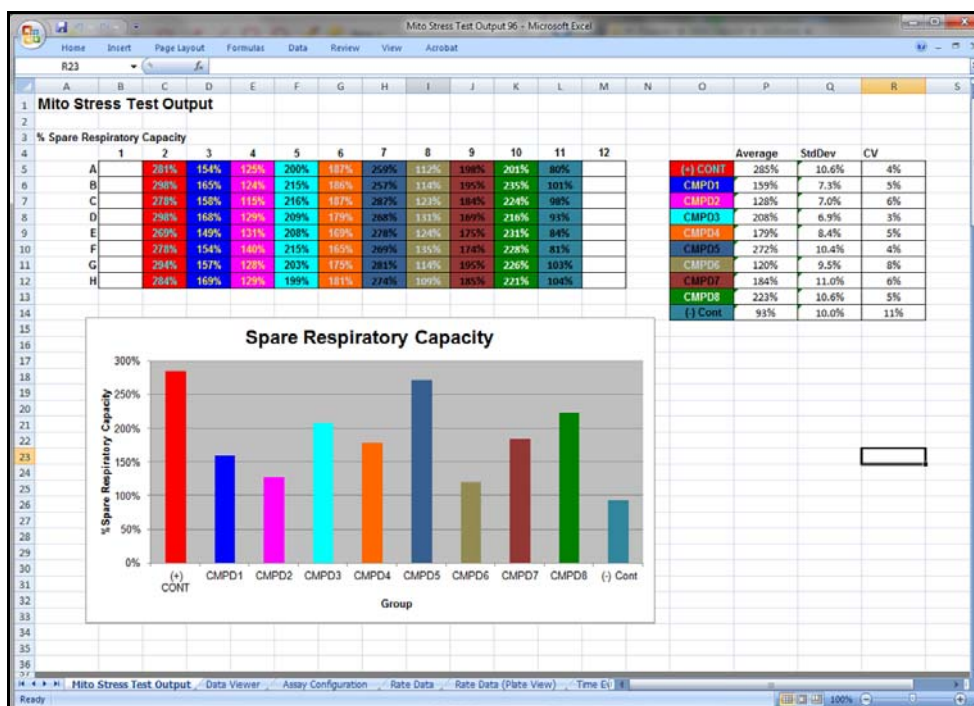


Figure 6.2
Spare Respiratory Capacity –
These data are presented as a % response from basal readings, with non-mitochondrial respiration subtracted out.

An important diagnostic of the bioenergetic status of a cell, particularly one which can experience a variable ATP demand, such as a neuron, is to determine the spare respiratory capacity - the ability of substrate supply and electron transport to respond to an increase in energy demand. Ideally this should be done by increasing oxidative phosphorylation directly, by activating an extra-mitochondrial ATPase, but since this is not always possible, an alternative is to add a carefully titrated concentration of protonophore, such as FCCP, sufficient to induce uncontrolled respiration. Under these conditions the mitochondria can still retain a considerable proton motive force, and are still able to generate ATP. The FCCP concentration is critical, since excess will collapse the proton motive force.

Coupling Efficiency

The coupling efficiency metric measures the amount of ATP turnover in the mitochondria, compared to a baseline reading.

The coupling efficiency data can be found by scrolling down to below the spare respiratory capacity data in the Mito Stress Test Output tab.

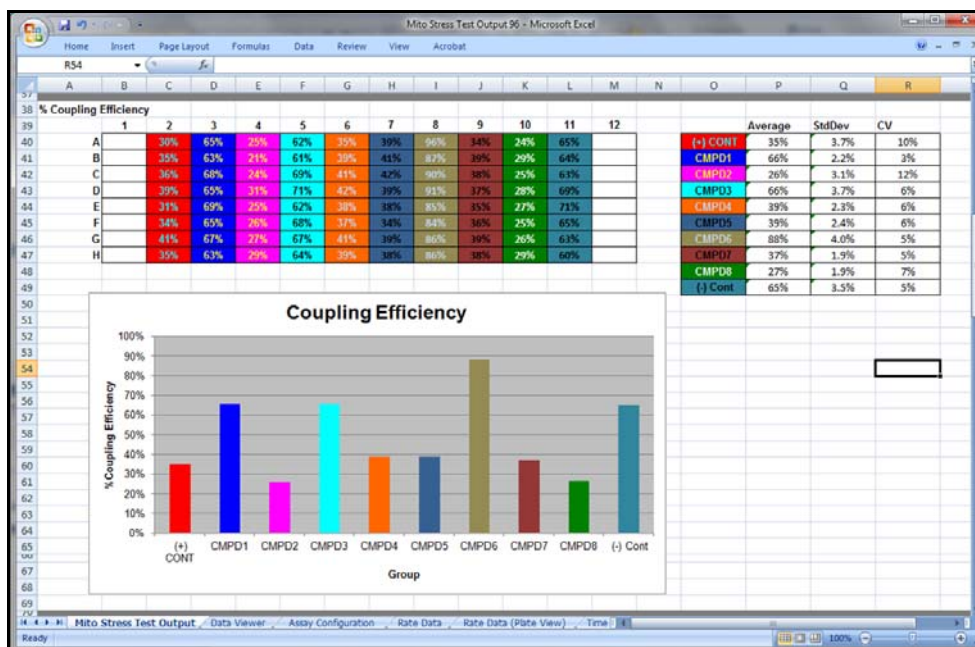


Figure 6.3
Coupling Efficiency – These data are presented as a % response from basal readings, with non-mitochondrial respiration subtracted out.

In a single experiment it is possible to quantify the most important bioenergetic diagnostics of the mitochondria within an intact cell. The proton current generated by basal respiration supplies the ATP synthase and the proton leak. An approximate measure of the mitochondrial ATP synthesis in the basal state can be obtained from the decrease in respiration by inhibiting the ATP synthase with Oligomycin, the residual respiration being ascribed to the proton leak. In practice, since ATP synthase inhibition results in a slight mitochondrial hyperpolarization, and the proton leak is voltage dependent, this approach can underestimate the ATP synthesis, and exaggerate the proton leak in the basal state.

The addition of Oligomycin shifts ATP production to glycolysis. To supply a cell with ATP at a rate comparable to that found during aerobic glycolysis requires that the pathway accelerate to more than tenfold. While most cell lines have sufficient glycolytic capacity, in some cells this may not be the case with the result that Oligomycin may induce an ATP crisis.

Basal Respiration

The basal respiration is the baseline oxygen consumption reading per well, before compounds are injected. The basal reading is determined by taking the 3rd basal measurement (the last measurement before compound injection). The basal reading is then used to baseline the responses to different compounds.

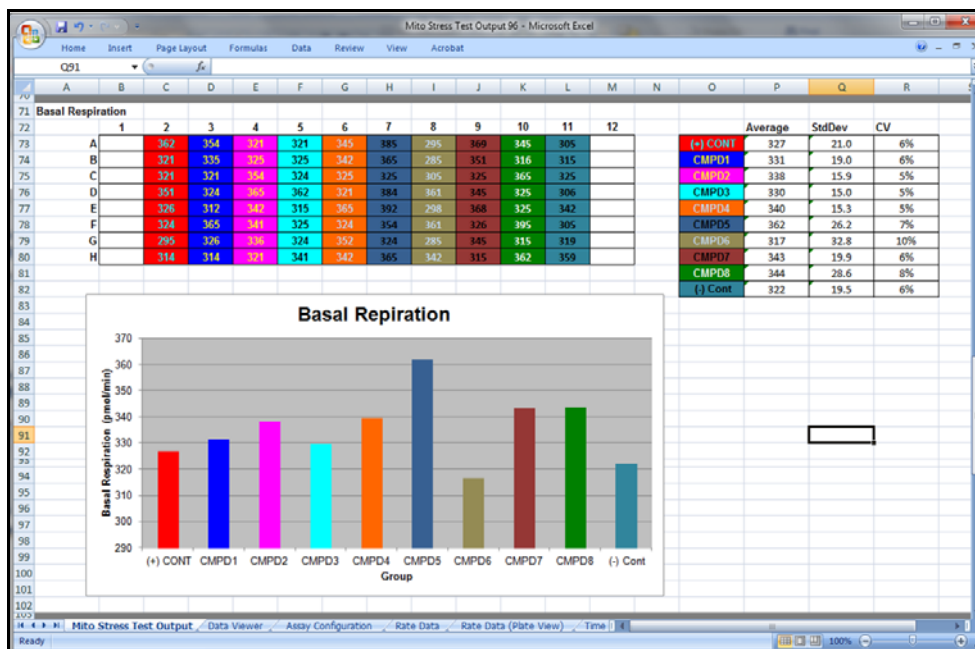


Figure 6.4
Basal Respiration – These data are presented as an absolute OCR reading (pMoles/min), taken from the third basal measurement.

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ETC Accelerator Response

The ETC Accelerator Response represents the maximum OCR measurement value after the ETC Accelerator (FCCP) injection. It is calculated by taking the maximum value from the 3 after injection measurements. This value is then used to calculate Spare Respiratory Capacity - represented here to provide the user an idea of maximum OCR readings seen in the assay.

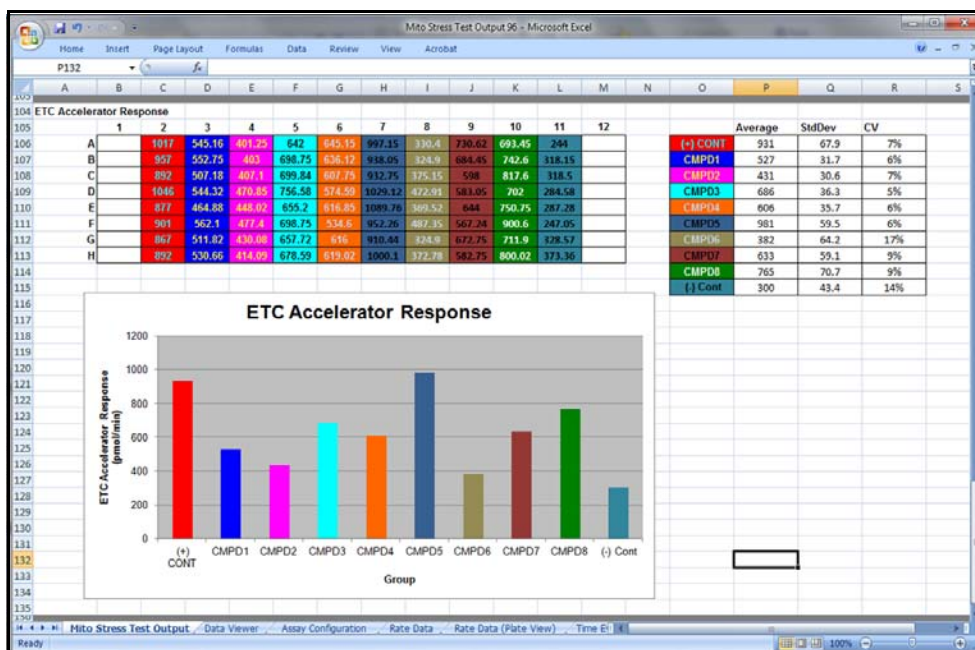


Figure 6.5
ETC Accelerator Response – These data are presented as an absolute OCR reading (pMoles/min), taken from the maximum OCR measurement after the ETC Accelerator injection.

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ATP Coupler Response

The ATP Coupler Response represents the minimum OCR measurement value after the ATP Coupler (Oligomycin) injection. It is calculated by taking the minimum value from the 3 measurements after injection. This value is then used to calculate Coupling Efficiency.

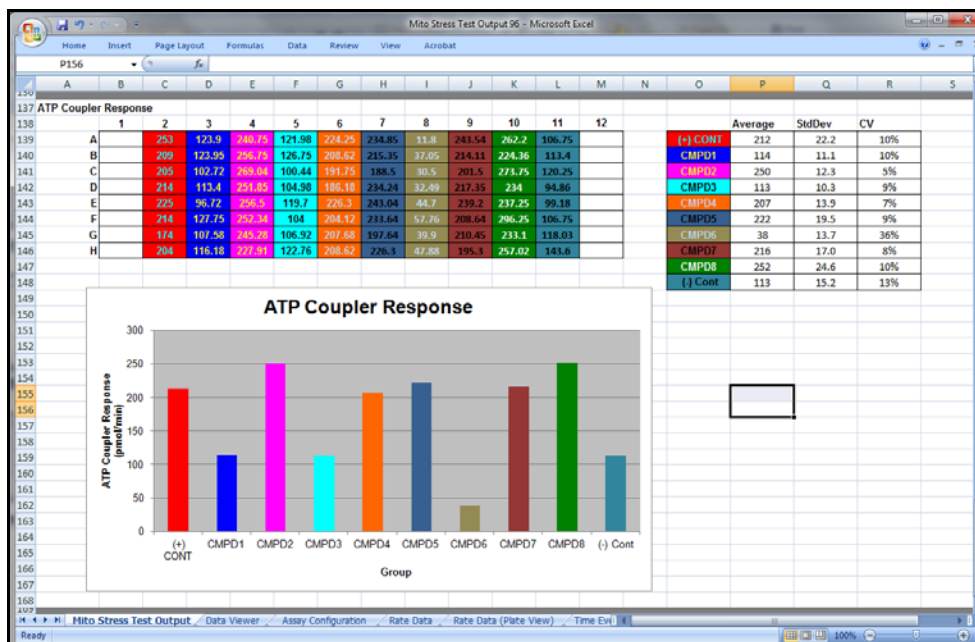


Figure 6.6
ATP Coupler Response – These data are presented as absolute OCR reading (pMoles/min), taken from the minimum OCR measurement after the ETC Accelerator injection.

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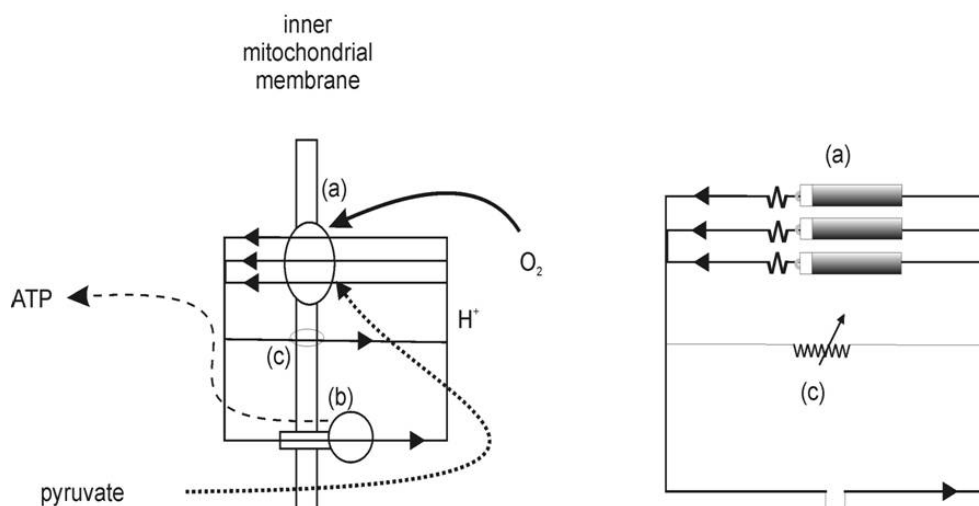
The Mitochondrial Proton Circuit

The mitochondrial proton circuit (Figure 6.7) is central to the multiple physiological functions of mitochondria. Three electron transport complexes, I, III, and IV pump protons across the inner mitochondrial membrane. In each case a decline in redox potential of the electrons passing through the complex, is coupled to the extrusion of protons from the matrix. Complexes I and III normally operate close to thermodynamic equilibrium, and can be induced to reverse under artificial conditions. Complex IV is irreversible. Complex II, of which succinate dehydrogenase is a part, is energetically incapable of pumping protons. The stoichiometry of the proton pumps is such that a total of 10 protons are extruded for each electron pair passing from NADH to oxygen. Therefore the rate of oxygen uptake by a cell (after correcting for any non-mitochondrial respiration) is the indicator allowing measurement of the proton current. The redox span across the electron transport chain is approximately 1100 mV, and provides an estimate of the maximal proton motive force across the inner membrane vary from 180 mV to 220 mV.

$$V = \Delta\psi \text{ (or strictly } \Delta p)$$

$$I = JH^+ = dO/dt \times H^+/O$$

Figure 6.7
The electrical
circuit analogy
of the proton
circuit.



In Figure 6.7, *Left*: the three proton pumping complexes of the electron transport chain (a) pump protons out of the mitochondrion generating a proton motive force. The proton circuit is completed by re-entry through the ATP synthase (b) coupled to the generation of ATP, or through a dissipative leak (c). *Right*: In the electrical analogy three electrical batteries, corresponding to Complexes I, III and IV generate a voltage and the electron circuit is completed by 'useful' work (lighting a bulb) or 'dissipative' work through the variable resistance.

The dominant pathway of proton reentry is via ATP synthase. In the absence of a proton motive force, the synthase acts as a proton pump hydrolyzing matrix ATP, and extruding protons from the matrix. However, the high proton motive force generated by the electron transport chain forces the complex to run in reverse, synthesizing matrix ATP, as protons are driven back through the complex. The electron transport chain proton

pumps together with the ATP synthase thus creating microscopic proton circuits across the inner mitochondrial membrane. The circuit has terms of potential, the proton motive force in millivolts, and flux, the proton current in nanomoles of protons per minute. This proton circuit is central to mitochondrial bioenergetics investigations. Experimentally distinct, but parallel experimental approaches are used to quantify the potential and flux components of the proton circuit, both with isolated mitochondria, and intact cells. The proton motive force is expressed in millivolts and is identical to the proton electrochemical potential which uses the thermodynamic unit of kJ/mole.

A useful conceptual model to consider is the proton circuit being analogous to a simple electrical circuit (Figure 6.7). An additional component is necessary to provide a more accurate model of mitochondrial bioenergetic function. All mitochondria possess an endogenous proton leak that is apparent also in mitochondria in situ within intact cells, and is thus not an artifact of isolation. In the absence of ATP synthesis the proton circuit is largely completed by the proton leak, which may serve as an important factor in limiting the proton motive force, therefore restricting leakage of single electrons from the electron transport chain to form the superoxide anion. The ATP synthase and the proton leak provide parallel pathways for proton reentry.

The electrical analogue can be quantified in terms of voltage, current and (by Ohm's Law) resistance or conductance. In the proton circuit the corresponding parameters are proton current (nmol H⁺/min/per unit of mitochondrial protein or cell number), proton motive force (in mV) and proton conductance (nmol H⁺/min/mV/per unit material).

Mitochondrial Proton Current (Respiration)

The tight coupling between electron transport and proton extrusion attests that, for a given substrate, the rate of mitochondrial oxygen utilization, with both the isolated organelles and intact cells, is an accurate measure of the total proton current. The many techniques available for monitoring the rate of oxygen utilization by mitochondria, all measure the activity of a single process, which is the transfer within complex IV of 4 electrons to a molecule of oxygen, to generate two molecules of water. Despite this, oxygen electrode experiments can be designed to obtain information on a wide variety of other processes within the mitochondria, such as substrate transport, dehydrogenase activity, electron transport through individual complexes, ATP synthase activity, and proton leak. Incubation conditions have to be designed such that the process to be investigated exerts significant control over the overall rate of electron transport.

In summary, when working with intact cells, or tissues, one should not assume that oxygen uptake is due to mitochondria only, since cells possess a variety of oxygenases. Therefore, when concluding a cell respiration experiment, it is advisable to determine residual respiration in the presence of electron transport inhibitors, such as Rotenone or Antimycin A, so as to optimize your assay results.

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